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Identification of Three Distinct *Polytomella* Lineages Based on Mitochondrial DNA Features

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ABSTRACT. *Polytomella* is composed of colorless green algae closely related to *Chlamydomonas reinhardtii*. Species in the genus have been used in diverse fields of biological research, most recently to study mitochondrial function and mitochondrial genome evolution in the Chlorophyceae, but the phylogenetic relationship between the various available taxa has not yet been clarified and it is not known whether they also possess fragmented mitochondrial genomes, as reported for *Polytomella parva*. We therefore examined *cox1* sequence from seven *Polytomella* taxa with the goal of establishing their phylogenetic relationships and relating this information to their mitochondrial DNA (mtDNA) fragmentation pattern. We found that the *Polytomella* isolates examined fall into three distinct lineages, two of which possess fragmented mitochondrial genomes. The third and earliest branching lineage, represented by *Polytomella capuana*, appears to possess an intact mtDNA. In addition, there is evidence for variation in both size and number of mtDNA fragments between various *Polytomella* isolates, even within the same lineage. The considerable amount of sequence divergence between lineages seems to correlate with the geographic origin of the strains, leading us to believe that greater amounts of sequence divergence could be uncovered by a broader sampling of *Polytomella*.

Key Words. Chlorophyceae, evolution, hybridization, mtDNA, phylogeny.

MOST currently available isolates from the genus *Polytomella*, a group of colorless green algae, were obtained and described by Pringsheim (1955). While using distinct names for the various isolates, Pringsheim acknowledged their similarity and referred to them as ''strains'' and ''species'' interchangeably. *Polytomella* taxa easily lend themselves to molecular studies since they are fast growing and easily cultured. In fact, members of the genus have at various times been proposed as model organisms in a variety of fields (Cooper et al. 1974; Gutiérrez-Cirlos et al. 1994; Pringsheim 1955). For example, *Polytomella* taxa have emerged as useful laboratory organisms for mitochondrial studies (e.g. Brumme et al. 1998; Fan and Lee 2002; Fan, Schnare, and Lee 2003; Pérez-Martìnez et al. 2001) in large part because they possess no cell wall or complex plastid materials, therefore facilitating cell breakage and mitochondrial isolation. *Polytomella* sp. (SAG 198.80) has been used in comparative studies of mitochondrial function with *Chlamydomonas reinhardtii*, a close relative (Nakayama et al. 1996; Pröschold et al. 2001), and it has been argued that the *Polytomella*/*Chlamydomonas* pair currently represents the best duo of photosynthetic and non-photosynthetic algae for comparative mitochondrial studies (van Lis, Gonzalez-Halphen, and Atteia 2005); an ongoing EST sequencing project focused on *Polytomella parva* will likely strengthen this position (Protist EST Program http://amoebidia.bcm.umontreal.ca/pepdb_c/pep.php).

Polytomella parva has been employed to explore mitochondrial genome evolution in green algae and was reported to possess a fragmented mitochondrial genome composed of linear 13.5- and 3.5-kb pieces (Fig. 1), each of which is flanked by homologous inverted repeat sequences of at least 1.3 kb (Fan and Lee 2002). The 3.5-kb DNA contains only one gene, *nad6*, which is missing from the 13.5-kb mtDNA. The *P. parva* mitochondrial genome can be directly compared to its *C. reinhardtii* counterpart in a number of ways: both are composed of linear DNA with long terminal inverted repeats and they share the same gene complement, with the exception of a reverse transcriptase-like- and two tRNA-coding regions not yet found in the mitochondrial genome of *P. parva*.

There are several other members of *Polytomella* maintained at the Sammlung von Algenkulturen Göttingen (SAG), but the genus as a whole is poorly studied. The objectives of our study were to provide a phylogenetic framework for the genus, determine whether other members of this group possess fragmented mitochondrial genomes and, if so, assess whether variation exists in size or number of mitochondrial DNA (mtDNA) pieces among these members.

MATERIALS AND METHODS

Strains and culture conditions. All *Polytomella* taxa were obtained from SAG and are listed in Table 1. Xenic cultures were made axenic by antibiotic treatment (Guillard 1973). Cells were grown for a few days at room temperature in *Polytomella* medium (0.1% tryptone, 0.2% yeast extract, 0.2% sodium acetate) supplemented with either spectinomycin sulfate (50 μg/ml) (MP Biochemicals, Aurora, OH) or erythromycin lactobionate (50 μg/ml) (Abbott Laboratories, Montreal, Canada); the elimination of contaminating microorganisms was established by microscopic examination and plating on LB medium. *Polytomella* taxa were then maintained in 10 ml of the standard growth medium at room temperature and transferred weekly.

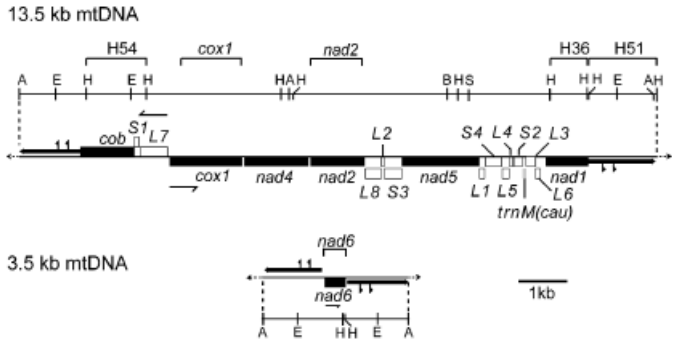

Fig. **1.** Partial restriction and gene map of the 13.5- and 3.5-kb mtDNAs of *Polytomella parva*. S1–S4 and L1–L8 are small subunit and large subunit rRNA-coding modules, respectively. Restriction sites shown are A, *Ava*I ; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. Fragments in brackets denote the location of probes used in this work. Thick solid arrows near the ends of the maps denote terminal inverted repeats; the two flags within these regions represent two direct subrepeats. Half arrows denote the direction of gene transcription. Dashed arrows at the very ends of the maps represent the predicted end structure of the 13.5- and 3.5-kb mtDNAs. Reproduced with modifications from Fan and Lee (2002).

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Table 1. *Polytomella* taxa used in this study.

Taxa employed	SAG number	Location of isolation
Polytomella caeca	63-1b	Unknown
P. papillata	63-2	Scotland, Glasgow
P. parva	63-3	England, Cambridge
P. magna	63-4	England, Cambridge
P. capuana	63-5	Italy, Capua
P. sp. I	198.80	Unknown
P. sp. II	63-10	Germany, Göttingen

Although both strain 63-10 and 198.80 are referred to as *P.* sp. in the SAG collection, they have been given different designations here to simplify discussion.

SAG, Sammlung von Algenkulturen Göttingen.

For DNA isolation, cells were grown in larger volumes with mild shaking until they reached the late log phase ($OD_{750} = 0.4$), before being harvested at 2,000 *g* for 10 min at 4 °C.

DNA extraction. Total DNA was extracted from *Polytomella* taxa according to the method of Aljanabi and Martinez (1997). Cell pellets were obtained from 250 ml of cell culture, resuspended in 3 ml of salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl, 2 mM EDTA, pH 8.0), and divided into three 1-ml samples for repelleting at 13,000 *g* for 30 s. The combined pellet was then resuspended in 400-µl salt homogenizing buffer and the rest of the procedure was performed as described in Aljanabi and Martinez (1997).

DNA blotting and hybridization. Vacuum blotting of agarose gels onto Hybond N+ membranes (Amersham, Buckinghamshire, UK) was performed using the Vacublot XL system (Amersham) according to the manufacturer's instructions. Probes used in this study (Fig. 1) were labelled and hybridized to samples using the AlkPhos Direct Labelling and Detection System (Amersham) following the manufacturer's instructions, except that hybridizations were carried out overnight at 60 °C. Detection was performed by exposing autoradiographic film (Fuji Super RX medical X-ray film) to the membrane. Blots were stripped for reprobing according to the manufacturer's recommendations and the absence of any residual signal was verified by exposing the blot to autoradiographic film.

DNA amplification. For *cox1* amplification from *Polytomella caeca*, *Polytomella papillata*, *P. parva*, *Polytomella magna*, *Polytomella* sp. I (*P.* sp. I), and *Polytomella* sp. II (*P.* sp. II), 25 µl PCR reactions were carried out using 12.5 nmol of the coxfor (5′-CWGCMYTRTTYGGIGGITTC-3′) and coxrev (5′-GCAC CMRTRCTYAAIACRTA-3′) primers, 1 µg of total DNA, and 22.5 µl of Platinum SuperMix High Fidelity (Invitrogen, Carlsbad, CA). DNA was denatured at 94 °C for 2 min, followed by an initial annealing at 50 °C for 3 min and elongation at 68 °C for 4 min. This was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 68 °C for 1 min.

A fragment of *cox1* from *Polytomella capuana* was obtained using the long walk PCR method of Katz et al. (2000), modified by Mary Ellen Boudreau and Camilla Nesbø, Dalhousie University (pers. commun.), which was anchored in the coding module for the mitochondrial subunit seven large subunit rRNA (primer L7: 5′-TCTAAGGTAGCGAAATTCCTTG-3′). Expanded *cox1* fragments from *P.* sp. II and *P. parva* were amplified to completely overlap with the sequence obtained from *P. capuana* using the largecox forward (5′-ATGCGTTGGYTKTATTCB-3′) and reverse (5′-AACATCGVCGWGGCATACC-3′) primers as described above.

Restriction enzyme digestion. Total DNA was digested overnight with either *Eco*RI, *Bam*HI, *Ava*I (10 U, 5 µg DNA, buffer 4, New England Biolabs, Ipswich, MA) or *Hin*dIII (10 U, 5 µg DNA, buffer 2, New England Biolabs). Samples were fractionated by agarose gel electrophoresis prior to blotting and hybridization with a compound probe derived from the *P. parva* 13.5 kb mtDNA (cloned fragments H54 and H36 and PCR products derived from *cox1* and *nad2*; see Fig. 1).

Cloning and sequencing of DNA fragments. PCR products were separated by agarose gel electrophoresis, extracted using the GenElute Gel Extraction Kit (Sigma, St. Louis, MO), and then cloned using the TOPO TA Cloning Kit (Invitrogen) with ampicillin (Sigma) as the resistance marker. Positive transformants were grown overnight in LB broth containing 50 µg/ml ampicillin. Plasmid DNA was then extracted using the GenElute Plasmid Miniprep Kit (Sigma) before being digested with *Eco*R I to screen for inserts. Colonies containing inserts of the appropriate size were sent to the Centre for Functional Microbial Genomics and Host Defence (see http://genomics.medicine.dal.ca/) (Halifax, Canada) for sequencing. At least two clones were sequenced with both forward and reverse primers for each *Polytomella* sequence reported.

Sequence analysis. GeneRunner (http://www.generunner.com) was used to edit and manipulate raw sequences. Multiple alignments of sequences were performed with CLUSTAL W (Thompson, Higgins, and Gibson 1994) using the default settings implemented on the BioEdit version 6.07 software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Where assigned gaps were not biologically plausible, alignments were manually edited using BioEdit. Maximum likelihood phylogenies were calculated using PAUP version 4.0b10 (Swofford 2003). New sequences reported in this work can be found in GenBank: *P. caeca* (DQ223136), *P. papillata* (DQ223137), *P. magna* (DQ223138), *P. capuana* (DQ221113), *P.* sp. I (DQ223139), *P.* sp. II (DQ221114). Other sequences used in generating phylogenies were obtained from *P. parva* (AY062933), *Chlamydomonas reinhardtii* (NC_001638), *Chlamydomonas eugametos* (NC_001872), *Scenedesmus obliquus* (NC_002254), and *Prototheca wickerhamii* (NC_001613).

RESULTS

Identification of mitochondrial DNAs. Total cellular DNA was isolated from the *Polytomella* taxa and subjected to agarose gel electrophoresis. Ethidium bromide staining revealed a band corresponding to the 13.5-kb mtDNA in the *P. parva* sample; similar-sized molecules were seen in the preparations from each of the other *Polytomella* taxa (Fig. 2A). The mitochondrial origin of the 13.5-kb band in all the samples was confirmed by its hybridization to a labelled *cox1* fragment derived from the 13.5-kb mtDNA of *P. parva* (Fig. 2B). The 13.5-kb mtDNA from *P.* sp. II and *P. capuana* hybridized significantly less to the *P. parva* probe than did the others, despite there being ample amounts of this DNA as seen in the ethidium bromide-stained gel (Fig. 2A).

To identify additional mtDNA species that may have been present in total DNA from the *Polytomella* taxa examined, further hybridization experiments were carried out using probe H51 (Fig. 2C), which spans 1.3 kb of a *P. parva* inverted repeat (Fig. 1). The inverted repeat sequence was selected as a probe because organisms with mitochondrial genomes consisting entirely of linear pieces typically have a defined terminal sequence (Nosek and Tomaska 2003). In *P. parva* the inverted repeat ends are conserved and hypothesized to be necessary for mtDNA replication; they should therefore be present in all the *Polytomella* 13.5-kb mtDNA species as well as any additional ones. For the *P. parva* DNA sample, this probe revealed the previously characterized 13.5- and 3.5-kb mtDNAs as well as bands of 2.1 kb, 1.8 kb, and additional hybridizing bands: one immediately below the 3.5-kb mtDNA and one corresponding to 1.7 kb. Apparently homologous DNA molecules, with some small size variation, were

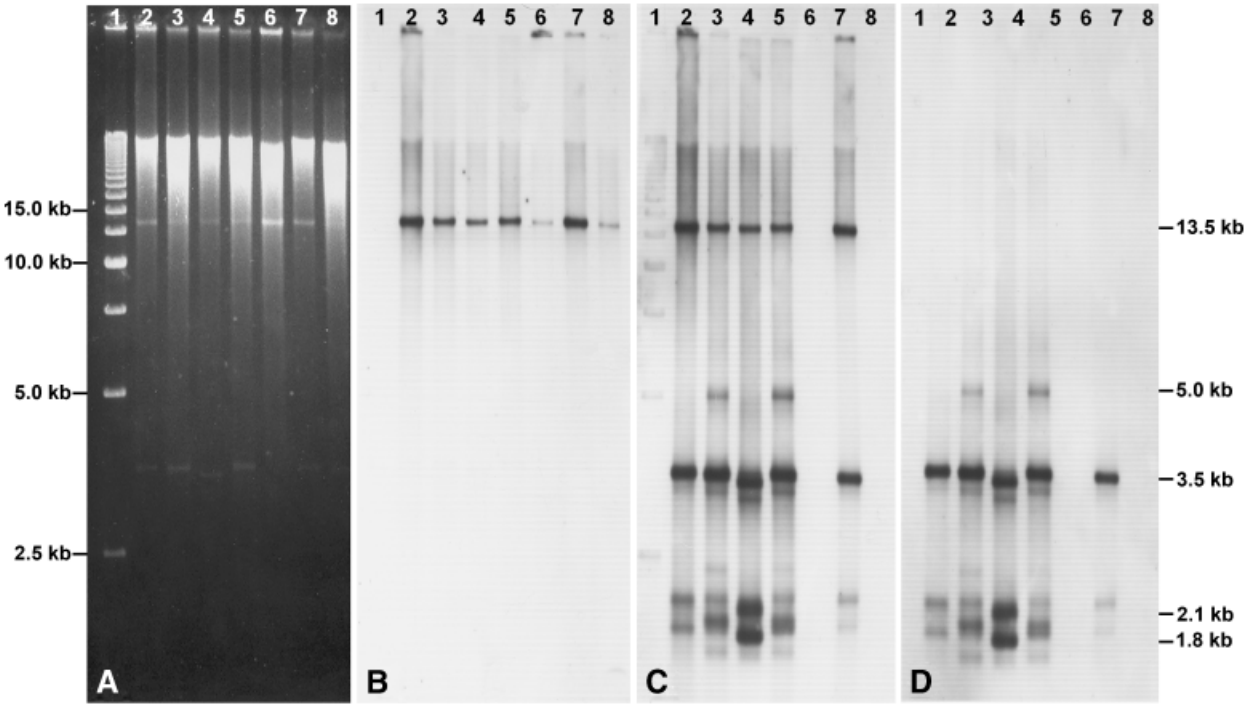

Fig. **2.** DNA blot hybridization analysis of total cellular DNA from seven *Polytomella* taxa following agarose (0.6%) gel electrophoresis. (**A**) ethidium bromide staining pattern. (B–C) DNA hybridization results using the *P. parva* (**B**) *cox1* probe, (**C**) H51 probe, and (**D**) *nad6* probe. Biorad 2.5–35 kb DNA ladder (lane 1), total DNA from *P. caeca* (lane 2), *P. papillata* (lane 3), *P. parva* (lane 4), P. *magna* (lane 5), *P. capuana* (lane 6), *P*. sp. I (lane 7), and *P*. sp. II (lane 8). Labels on the left denote the size of selected markers, while those on the right identify the size of selected hybridizing bands relative to the linear size markers.

detected in all the DNA samples showing hybridization to the H51 probe. *Polytomella papillata* and *P. magna* were found to possess H51-hybridizing fragments of 5- and 2.5 kb, not present in other strains (Fig. 2C). All H51-hybridizing fragments in the *P. papillata* sample were shown to be linear based on their co-migration with linear markers under different electrophoresis conditions (Fig. 3).

Polytomella capuana and *P*. sp. II, whose 13.5-kb mtDNA showed reduced hybridization to the *cox1* probe relative to the other samples, did not hybridize to the *P. parva* inverted repeat sequence (Fig. 2C). However, lowering the hybridization stringency and exposing the autoradiographic film overnight allowed a faint hybridization pattern to be detected from *P*. sp. II, which appeared identical to the pattern observed from *P*. sp. I and *P. caeca*; no signal was observed from *P. capuana* under these conditions (MAM. & RWL., pers. observ.). For *P. capuana*, we were unable to detect a band corresponding in size to the 3.5-kb mtDNA of *P. parva* by ethidium bromide staining, despite readily observing such a band in all other *Polytomella* taxa examined (Fig. 4).

Hybridizations with *nad6*. Previous work (Fan and Lee 2002) suggested that *P. parva* mtDNA fragments in addition to the 3.5-kb mtDNA could harbour *nad6* sequence. Hybridization experiments with the *P. parva* probe confirmed this observation for *P. parva* and also demonstrated this for other *Polytomella* taxa (Fig. 2D); all fragments that hybridized to the inverted repeat probe (H51) also hybridized to the *nad6* probe except for the 13.5-kb mtDNA species.

Sequence analysis. An 894-bp fragment from *cox1* corresponding to nucleotides 223–1116 in the *P. parva* gene was amplified, cloned, and sequenced from *P. caeca*, *P. papillata*, *P. parva*, *P. magna*, *P*. sp. I, and *P*. sp. II. Attempts to PCR amplify

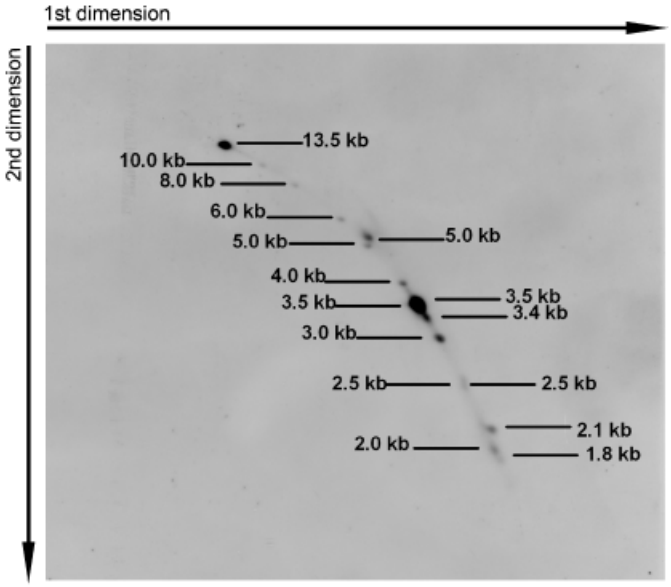

Fig. **3.** Hybridization analysis of total cellular DNA from *P. papillata* subjected to two-dimensional gel electrophoresis. DNA from *P. papillata* was electrophoresed (0.4% agarose at 1 V/cm for 18 h) in a lane containing labelled linear ladder (Fermentas, Burlington, Canada). The lane was cut out and subjected to gel electrophoresis (1% agarose at 2 V/cm for 13 h) in an orthogonal plane. The gel was then blotted onto a nylon membrane and probed with H51. Labels on the left refer to the linear markers while those on the right refer to DNAs hybridizing to the H51 probe.

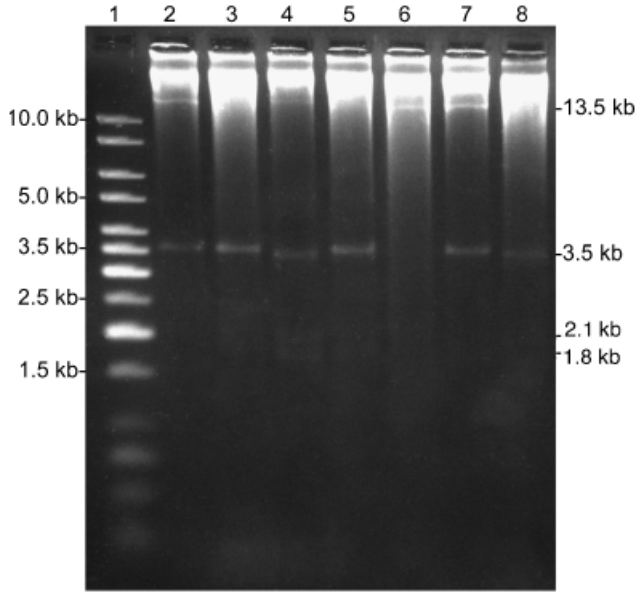

Fig. **4.** Agarose (0.8%) gel electrophoresis of total cellular DNA from seven *Polytomella* taxa. Biorad 2.5–35 kb DNA ladder (lane 1), *P. caeca* (lane 2), *P. papillata* (lane 3), *P. parva* (lane 4), P. *magna* (lane 5), *P. capuana* (lane 6), *P*. sp. I (lane 7), and *P*. sp. II (lane 8). Labels on the left denote the size of selected markers, while those on the right denote selected mtDNAs.

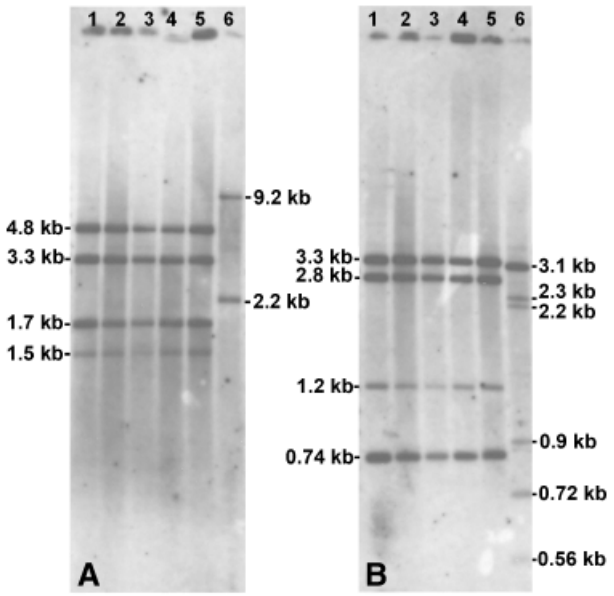

Fig. **5.** DNA blot hybridization analysis of total cellular DNA from six *Polytomella* taxa digested with restriction endonucleases and hybridized with a compound probe prepared from *P. parva* mtDNA as described in the Materials and Methods. (**A**) *Eco*RI, *Bam*HI, and *Ava*I. (**B**) *Hin*dIII. *P. caeca* (lane 1), *P. papillata* (lane 2), *P. parva* (lane 3), *P. magna* (lane 4), *P*. sp. I (lane 5), *P*. sp. II (lane 6). Labels on the left correspond to the expected size of *P. parva* restriction fragments, based on available mitochondrial sequence data, while those on the right identify the restriction fragments of *P*. sp. II. Note that the pattern for *P*. sp. II (lane 6) is markedly different from those of the other taxa.

cox1 sequence from *P. capuana* using the same coxfor and coxrev degenerate primers failed to produce a product under a variety of annealing temperatures and primer/template combinations. Long-walk PCR, however, successfully amplified a 768-bp fragment of *P. capuana cox1*, corresponding to nucleotides 1–768 in the *P. parva* sequence. Multiple alignment of the 546 nucleotides common to the seven *Polytomella* taxa revealed three distinct sequence types, one from *P. caeca*, *P. papillata*, *P. parva*, *P. magna*, and *P*. sp. I, and one each from *P. capuana* and *P*. sp. II. Compared to *P. parva*, the sequences from *P. capuana* and *P*. sp. II showed 71% and 84% identity at the nucleotide level and 94% and 99% identity at the predicted amino acid level, respectively.

Because of the possibility for PCR artifacts, it was of interest to assess the apparent similarity of mtDNA sequence from *P. caeca*, *P. papillata*, *P. parva*, *P. magna*, and *P*. sp. I using a PCR-independent method. To this end, restriction enzyme digestion patterns of the 13.5-kb mtDNA were obtained by hybridization with probes spanning the 13.5-kb mtDNA of *P. parva* but not including the inverted repeat sequence. The twelve restriction sites surveyed, which were located in diverse regions of the 13.5-kb mtDNA, including the inverted repeat ends, rDNA, and various protein-coding genes (Fig. 1), were all cleaved (Fig. 5). DNA from *P*. sp. II, included as a negative control, revealed a banding pattern distinct from the pattern observed for the other five taxa.

A 768-bp *cox1* sequence from *P. parva*, *P*. sp. II, and *P. capuana* corresponding to nucleotides 1–768 in the *P. parva* gene was used along with homologous sequences from other selected green algal representatives in the Chlorophyceae (*C. reinhardtii*, *Chlamydomonas eugametos*, *and Scendesmus obliquus*) and Trebouxiophceae (*Prototheca wickerhamii*) to generate a phylogeny using the maximum likelihood method. The branching order obtained within the *Polytomella* group is supported by strong bootstrap values (Fig. 6). Robust trees showing the same topology for the *Polytomella* lineages were obtained using the maximum parsimony, neighbor joining, and minimum evolution methods (MAM., unpubl. data). These data identify *P. capuana* as the earliest branching of the *Polytomella* taxa surveyed.

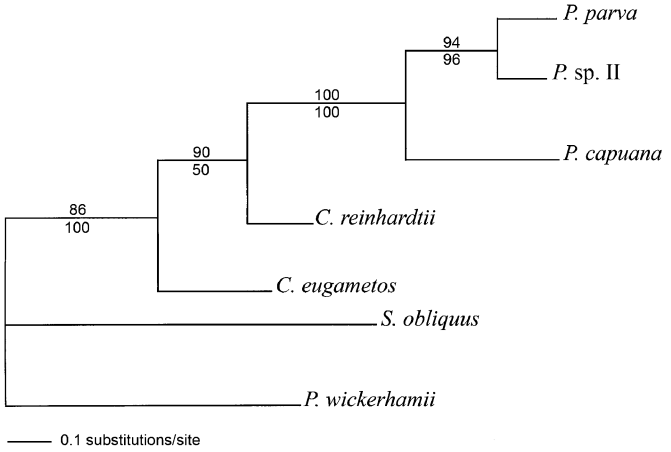

Fig. **6.** Phylogenetic analysis of a *cox1* fragment from *Polytomella parva*, *P*. sp. II, *P. capuana*, *Chlamydomonas reinhardtii*, *Chlamydomonas eugametos*, *Scenedesmus obliquus*, and *Prototheca wickerhamii* using the maximum likelihood method under a GTR+G+I model and γ-distributed rate-variation (discrete γ, estimated from the data by ML with six categories). All taxa are members of the Chlorophyceae with the exception of *P. wickerhamii*, which is in the Trebouxiophyceae. Upper bootstrap values were obtained using all three codon positions and correspond to the tree shown, while lower bootstrap values were obtained by including only the first and second positions in the analysis. Branch lengths were calculated using all three codon positions.

DISCUSSION

Identification of *Polytomella* mitochondrial DNAs. One of the goals of this study was to determine if all of the *Polytomella* taxa examined possess fragmented mitochondrial genomes, as is the case for *P. parva*. Using hybridization analyses with total cellular DNA fractions we showed that all these taxa, except *P. capuana*, possess fragmented mitochondrial genomes. The presence of an unfragmented mitochondrial genome for *P. capuana* is supported by the absence of mtDNAs other than the 13.5 kb mtDNA in ethidium bromide-stained gels of total DNA. This conclusion is further supported by the recovery of only a 13.5-kb DNA species from a DNAse treated mitochondrial fraction from this taxon (Borza, T. & RWL., unpubl. data). For *P. papillata*, all the fragments hybridizing to the *P. parva* mtDNA probes appear to be linear molecules; apparently homologous mtDNA fragments in other members of the genus are presumed to be linear as well.

For *P. caeca*, *P. papillata*, *P. parva*, *P. magna*, and *P.* sp. I, a probe derived from *P. parva nad6* hybridized to all mtDNA species that were identified earlier with the inverted repeat sequence from *P. parva*, except for the 13.5-kb fragment. A signal corresponding to the 3.5-kb mtDNA of *P. parva*, which contains an apparently functional copy of *nad6* in that taxon, was obtained from *P. caeca*, *P. papillata*, *P. magna*, and *P.* sp. I. In *P. parva*, this mtDNA is thought to be autonomously replicating because it possesses the same set of inverted repeat ends as the 13.5-kb mtDNA (Fan and Lee 2002). The conclusion that the other *nad6*-hybridizing fragments harbor *nad6* sequence is an interesting one considering that some of the fragments are smaller than 3.5 kb and are not big enough to contain both a full *nad6* and two complete inverted repeat ends. These smaller *nad6*-hybridizing fragments, some of which were previously identified in the mtDNA of *P. parva* (Fan and Lee 2002), have been observed across multiple DNA isolations in all of these taxa. This may indicate that they are stably inherited, but it is also possible that they are continuously generated. Irrespective of their origin, it seems certain that all of the *nad6*-hybridizing fragments are somehow derived from each other because these DNAs undoubtedly descended from a mitochondrial genome with a single copy of *nad6*. Finally, while unequal crossing over between two 3.5-kb mtDNAs could potentially account for the 5- and 2.5-kb fragments of *P. papillata* and *P. magna*, a specific hypothesis for the origin of the smaller *nad6*-hybridizing fragments is difficult to formulate without more sequence information.

For *P.* sp. II and *P. capuana*, no hybridization to the *nad6* probe was observed, presumably because of extensive sequence divergence. We expect *nad6* sequence to be located in the 3.5 kb and associated mtDNAs of *P.* sp. II, where it is found in *P. caeca*, *P. papillata*, *P. parva*, *P. magna* and *P.* sp. I. For *P. capuana*, whose mitochondrial genome is unfragmented, the *nad6* gene is likely located in the 13.5-kb mtDNA although it could also have been transferred to the nucleus.

Mitochondrial sequence diversity in the *P. parva* group. *Polytomella caeca*, *P. papillata*, *P. parva*, *P. magna*, and *P.* sp. I are very closely related and possibly conspecific. All 546 nucleotides of a corresponding PCR fragment of *cox1* from each of these taxa were identical. However, a *cox1* sequence from *P.* sp. I (Antaramian et al. 1996) was previously reported (GenBank Accession no. AF286057) and it differs from the sequence described here by two adjacent non-synonymous substitutions at nucleotides 422 and 423 relative to the *cox1* sequence of *P. parva* (GenBank Accession no. AY062933). The two amino acids corresponding to these positions and predicted by our sequence are conserved in all other green algae examined, including *P. capuana* and *P.* sp. II. Restriction site analysis also supports the apparent identity of mtDNA sequence from *P. caeca*, *P. papillata*, *P. parva*, *P. magna*, and *P.* sp. I. Nevertheless, these taxa do possess differences in the composition of their mtDNA fragments, which indicates that they are not absolutely identical. Also, a sequence for *cob* from *P.* sp. II is available (GenBank Accession no. U87396), which differs from the published *P. parva* sequence (GenBank Accession no. AY062933) by three nucleotides. These differences need to be confirmed. Finally, it would be useful to determine whether mating can occur between pairs of taxa from this group of five polytomellas, but such experiments would be complicated by the fact that sexual reproduction in this genus appears to be homothallic and is rare under laboratory conditions (de la Cruz and Gittleson 1981; Moore and Cushing 1979).

Phylogeny and taxonomy of *Polytomella*. Phylogenetic analysis of the *cox1* sequences obtained from *P. parva*, *P.* sp. II, and *P. capuana* yielded a tree that was generally well supported. The phylogeny strongly suggests that *P.* sp. II is more closely related to *P. parva* than it is to *P. capuana*, a conclusion which is consistent with the hybridization intensity of *P. parva* mtDNA probes with the *P.* sp. II and *P. capuana* mtDNAs. The sister relationship of the *Polytomella* group with the *C. reinhardtii* lineage is consistent with other analyses (Nakayama et al. 1996; Pröschold et al. 2001) supporting *P. parva* as an early branching member of the ''*Volvox* clade'' sensu Nakayama et al. (1996) and as a sister lineage of the ''*Reinhardtii* clade'' sensu Pröschold et al. (2001). The analysis of sequence data presented here and morphological/physiological data discussed by Pringsheim (1955) are consistent with the *Polytomella* taxa forming a natural group within the *Volvox* clade although this cannot be taken as formally proven. The relationships between the non-*Polytomella* taxa in Fig. 6 are consistent with other analyses (Friedl 1997; Nakayama et al. 1996; Pröschold et al. 2001).

Polytomella capuana, which appears to possess an unfragmented linear mitochondrial genome, was identified as the earliest branching of the *Polytomella* taxa examined here. Considering that the ancestral mitochondrial genome of the *Volvox* clade is proposed to be a single linear mtDNA molecule (Laflamme and Lee 2003), the most parsimonious explanation is that fragmentation of the *Polytomella* mitochondrial genome occurred within the *Polytomella* clade after the divergence of *P. capuana* but prior to the divergence of *P. parva* and *P.* sp. II.

The relationships inferred for the genus *Polytomella* seem especially sensible when available biogeographical information is taken into account; *P. parva*, *P. papillata*, and *P. magna* were all isolated in the United Kingdom while *P.* sp. II was recovered in Germany and *P. capuana* was isolated from Italy. For *P. caeca* and *P.* sp. I, the locations of isolation remain unknown but the present data suggest that they may both originate from the United Kingdom. Considering the degree of sequence divergence between taxa with known locations of isolation, which increases with the distance between the sites of origin, we would expect even greater divergence by including taxa isolated from a wider geographical area. However, the apparent relationship between the mtDNA sequence data and available biogeographic data could be an artifact of undersampling. Clearly, molecular analyses of the type described here on additional widely sampled *Polytomella* isolates are justified.

Finally, *P.* sp. II is currently an unnamed strain in the SAG collection although the data presented here clearly demonstrate that it represents a distinct lineage within the *Polytomella* group and may therefore warrant a name. This isolate (SAG 63-10) was originally designated *Polytomella piriformis* by Pringsheim (unpublished) and the apparently identical strain UTEX 1296 currently retains this name.

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